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Cardiovascular Protective Agents and their Biochemical Role in Endothelial Cell Senescence

By
Nicholas Glen Boullard

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College

Oxford
March 2014

Approved by

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DEDICATION

I would like to dedicate this thesis to my parents, Danny James Boullard, Wanda Ardoin Bailey, and Jimmy Leo Bailey. Without these three people, I wouldn't have been able to graduate high school, let alone join one of the top Honors College programs in the country. They laid a foundation of hard work and sacrifice that I continue to build off of every day, and for that, I thank them. I wouldn't be on the path I am today without them.

ABSTRACT

NICHOLAS G. BOULLARD: Cardiovascular Protective Agents and their Biochemical Role in Endothelial Cell Senescence

Cellular senescence is a slow, biological process of aging that involves the accumulation of various changes to the internal environment of a cell, most notably the buildup of acidic B-galactosidase. These changes, both structural and molecular in nature, disable metabolism of many cellular processes and eventually induce apoptosis. As cells divide, the ends of chromosomes, telomeres, slowly shorten. Once telomere shortening reaches critical levels, those chromosomes may no longer replicate properly, leading to cellular process complication and apoptosis. Telomerase is a reverse transcriptase that adds nucleotides to telomeres, elongating them and successfully delaying endothelial cell senescence. Endothelial cells serve an irreplaceably important part in our body as the thin layer of cells that line the furthest interior surface of our blood vessels. They are the site of reception between molecules circulating in the blood. These endothelial cells are the sites of many physiological processes; especially blood clotting, vasoconstriction, vasodilation, and inflammation. The biochemical processes of the proteins in the kinin-kallikrein system drive the responses of inflammation, blood pressure regulation, and blood coagulation via many mediators. One of the most important products, bradykinin, is generated from human plasma when prolylcarboxypeptidase (PRCP) converts prekallikrein (PK), bound to high molecular weight kininogen (HK) on the negatively charged surface of human endothelial cells. This bradykinin drives vasodilation as it produces nitric oxide and prostacyclin. These vasodilative properties increase oxygen delivery and promote further angiogenesis and delay cellular death. Keeping this in mind, PRCP, as an indirect vasodilator, and basic fibroblast growth factor (FGF-2), as a promoter for angiogenesis, should both promote cell survival and delay endothelial cell senescence. As a control, B-actin and glyceraldehyde-3-phosphate dehyedrogenase (GAPDH) were used; B-actin is prevalent in all cells throughout their life as an integral cytoskeletal actin, and GAPDH is an important step in the process of glycolysis, the breakdown of glucose for energy and carbon molecules. In our results, we found that the activity of HK-PK peaked at Passage 18, and to support that, we found that the converting enzyme, PRCP, shared the same peak in activity. Our results were supported when we found that nitric oxide (NO), a downstream product of the PRCP-catalyzed HK-PK system, and endothelial nitric oxide synthase (eNOS), responsible for another method of NO production shared the same peak in activity as PRCP. Human telomerase reverse transcriptase (hTERT) mRNA concentration was examined alongside B-galactosidase (B-gal) cell staining, a universal marker for

cellular senescence. Our results show that the decline in hTERT precedes the onset of endothelial cell senescence, and that the buildup of B-gal is a sufficient indicator for the arrival of endothelial senescence.

This study has emphasized the role that HK and PK play in cellular health and the delay of endothelial cellular senescence. We propose that nitric oxide and FGF-2 delay endothelial cell senescence by slowing the age-related degradation of telomerase.

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LIST OF ABBREVIATIONS

Plasma Kallikrein Kinin System	(KKS)
Prekallikrein	(PK)
High Molecular Weight Kininogen	(HK)
Prolylcarboxypeptidase	(PRCP)
Bradykinin	(BK)
Human Pulmonary Endothelial Artery Cells	(HPAEC)
Constitutive Bradykinin B2 Receptor	(B2R)
Inducible Bradykinin B1 Receptor	(B1R)
Nitric Oxide	(NO)
Fibroblast Growth Factor-2	(FGF-2)
Human Telomerase Reverse Transcriptase	(hTERT)
Endothelial Nitric Oxide Synthase	(eNOS)
Glyceraldehyde 3-Phosphate Dehydrogenase	(GAPDH)

I) INTRODUCTION

A) Telomeres

In the 1930, a geneticist named Barbara McClintock noted that DNA, as the carrier of our genetic information, would need to have some kind of protective mechanism at its ends in order to prevent the fusing and/or breakage of chromosomes during mitosis (McClintock, 1941). These protective caps went on to be called “telomeres,” Greek for “end parts.” The first studies on telomeres proved that they existed to provide stability and successful segregation of genetic information within cellular division. As time progressed, great advances were made in understanding telomeres. Telomeres are guanine-rich repetitive DNA that are maintained by their respective reverse-transcriptase enzyme, telomerase. DNA’s telomere cap is composed of a single strand between 50 and 300 nucleotides, which has been proposed to “fold back onto the duplex telomeric DNA,” forming a “T-loop” structure (Aubert and Lansdorp, 2008). As cells divide, telomeric DNA is lost little by little. Eventually, the closed loop unfolds, and the longer DNA strand is left uncopied. Over time, the telomere loss accumulates (Figure 1). Normal replication is the main cause for telomere loss due to the fact that DNA replication id bidirectional and DNA polymerases are unidirectional. DNA polymerases must initiate from a primer, and because of this, each round of DNA replication must leave up to two hundred base pairs unreplicated at the 3’ end of the DNA (Shampay and Szostak, 1984). For a while, the telomeres are seen as salvageable, and reverse transcription by telomerase adds more nucleotides to the telomeres, extending their life. Once too many broken ends build up, the telomeres are seen as unsalvageable, and p53 sends the cell into a state of “replicative senescence” to await the eventual apoptosis (Shen et al., 2013).

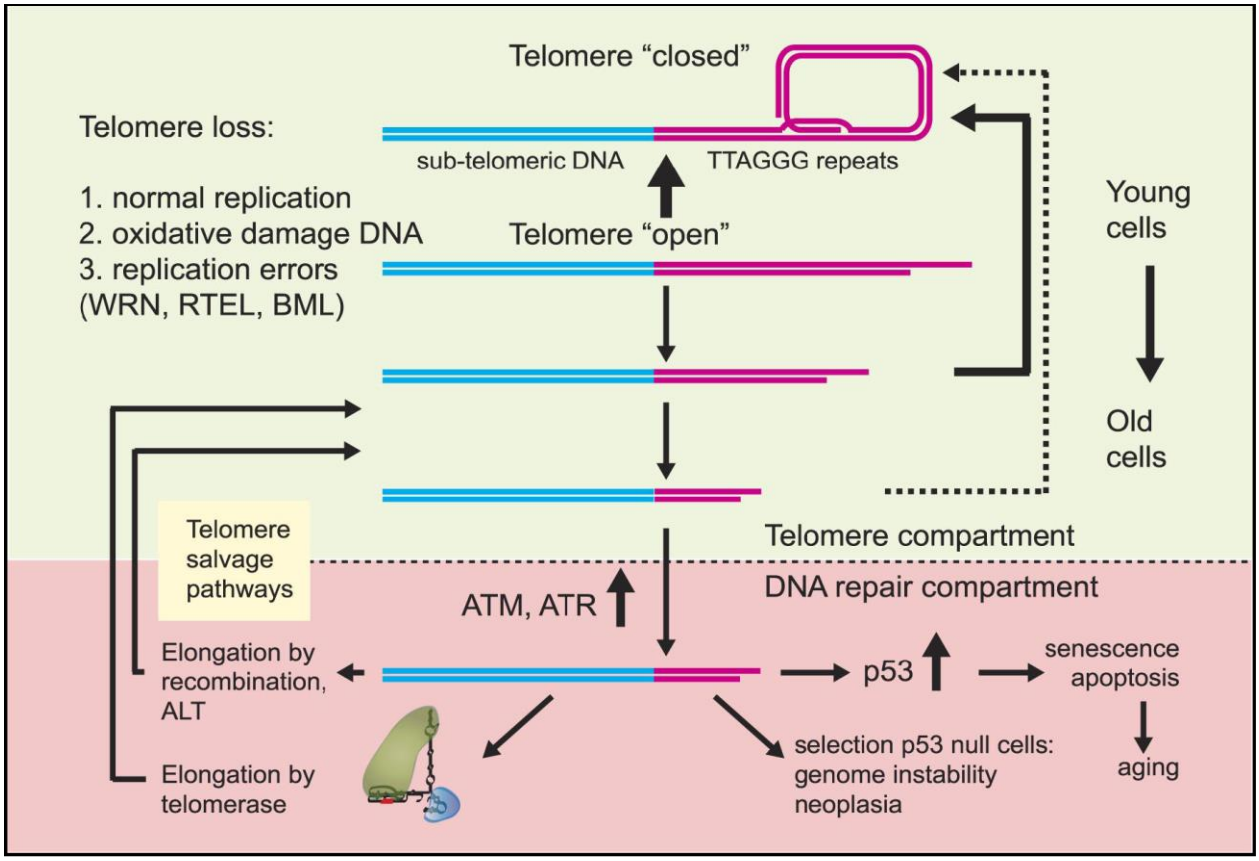


Figure 1: This diagram shows the primary factors that affect telomere length in human somatic cells. The telomeres in younger somatic cells have long G-rich nucleotides that favor the folded structure. As the cells age, and telomere length decreases, the loop eventually unfolds. Once the ends become too short, there are various methods of DNA repair, most notably telomerase, which varies depending on the cell type. Eventually, too many short and broken ends will build up and DNA damage signals, such as p53, will signal senescence, and eventually apoptosis. (Aubert and Lansdorp, 2008)

B) Cellular Senescence

As we age, all of our biological processes and function lose their efficiency. Age is not a direct cause of death, but the decreased function of our bodies, brought on by age, is. In the early 1960s, Leonard Hayflick observed that a culture of human tissue, in vitro, stopped dividing once the culture had reached an old age via numerous cellular divisions. He hypothesized that this phenomenon could be used to study human aging at the cellular level (Hayashi et al., 2014). Cells divide in order to counterbalance healthy cell loss and to repair bodily injury, but certain cells divide far more frequently than others. It is not uncommon to see age take place without much cellular turnover, but with the correlation between cellular fitness to healthy telomeres provides a sense of order to this conundrum. For example, telomeric DNA damage has been recorded in different human tissues that have aged and no longer undergo mitosis, such as the brain, heart, and kidneys. Reactive oxygen species (ROS), and as well as the signaling pathways of various oncogenes, have been documented to damage telomeric DNA effectively (Hayashi et al., 2014). As a result, it can be concluded that telomeric DNA damage is capable of being replication-independent, and that environmental predispositions to DNA damage would only exacerbate the decline in telomere length. The connection between telomeres and aging was established in 1986 when Cooke and Smith realized that the telomere repeats were significantly longer in sperm cells than adult somatic cells. Several studies brought the conclusion that somatic cells are simply unable to maintain telomere length well, and for the first time, the aging of cells could be directly linked to genomic DNA (Aubert and Lansdorp, 2008).

C) Nitric Oxide

One of the most outstanding studies involving cell senescence explores telomerase's rejuvenation of telomeric DNA and its involvement in delaying cellular senescence. Upon further investigation, it is observed that the vasodilatory molecule, nitric oxide (NO), is involved in this reconstruction. It has been found that NO can activate telomerase and delay endothelial cell senescence (Donnini et al., 2010). The nitric oxide donor, S-nitroso-penicillamine (SNAP), has been found to significantly reduce endothelial cell senescence. Likewise, telomerase inactivation precedes cellular aging, but nitric oxide has been identified as being able to prevent age-related downregulation of telomerase activity, delaying endothelial cell senescence (Vasa et al., 2000). The exact mechanism with which nitric oxide prevents the downregulation of telomerase has yet to be determined, but it is clear that telomerase can be modulated. Some researchers believe that nitric oxide might react with tissue-derived oxygen radicals to reduce oxidative stress, a factor that is a powerful accelerator of endothelial cell senescence. Others believe that there may be a transcriptional/post-transcriptional mechanism by which nitric oxide could upregulate telomerase production. Regardless, it is known that endothelial protection is one of the main functions of nitric oxide (Vasa et al., 2000).

D) The Circulatory System

As a postmitotic organ, the heart serves as an excellent subject for observing the degradation of telomeric DNA as it coincides with the approaching senescence. The heart, part of the circulatory system, is a "four chambered, double pump" with the sole purpose to create the pressure required in order to drive our blood to the lungs, limbs, and the rest of the body (Fox,

1999). The circulatory system, as a whole, serves three purposes: “transportation, regulation, and protection.” All metabolites, nutrients, and waste that the cells of the human body transport are done so via the cardiovascular system. All of the hormonal and temperature regulation done by the body is accomplished through the blood. In the event of an injury or invasion of foreign microorganisms and toxins, the circulatory system can either respond with clotting or leukocytes (Fox, 1999).

The other half of the circulatory system, the system of blood vessels, is a tubular network that carries the oxygen-rich blood from the heart to the tissues of the body in arteries before sending the oxygen-depleted blood back to the heart in veins. Arteries and veins are separated by microscopic, extremely thin capillaries, which bridge the arterial system to the venous system. This bridge is where the fluids, nutrients, and waste are exchanged while the blood flows from the smallest arteries, arterioles, to the smallest veins, venules. This “tree of progression” works in a nonstop, fluid motion from the heart, to the arteries, the arterioles, the capillaries, the venules, the veins, and then the heart again, all to be replenished (Fox, 1999). The walls of arteries and veins are composed of three layers, also known as tunics (Figure 2). From the outside, to the inside, they are “tunica externa, tunica media, and tunica interna.” This brings us to the tissue of our focus: the endothelium. The endothelium is the innermost layer of the innermost tunic (Fox, 1999). This squamous epithelium is the innermost layer of all blood vessels and it is the focus of production of nitric oxide and the cellular senescence which it delays.

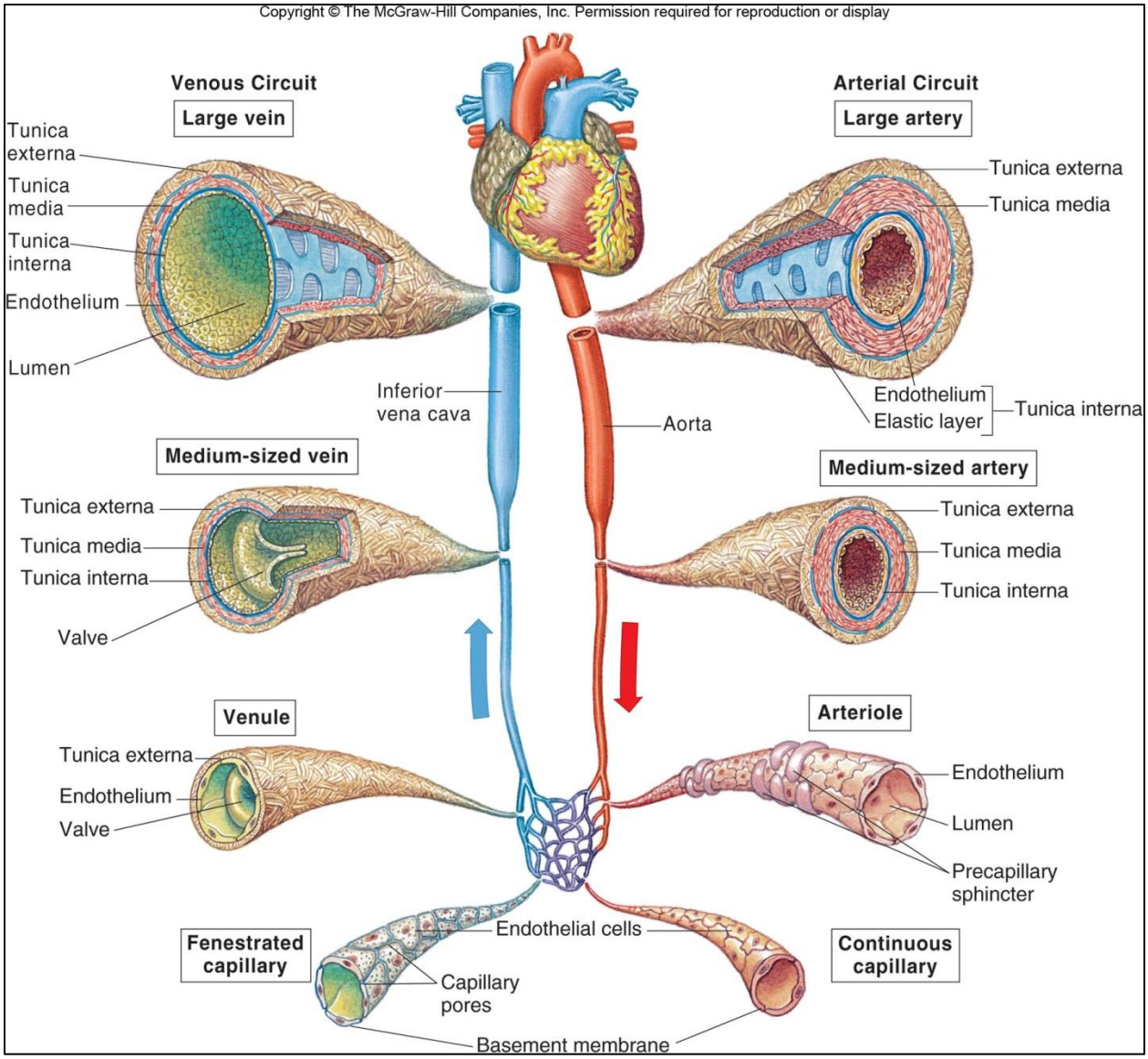


Figure 2: The system of blood vessels to and from the heart (Fox, 1999).

E) Endothelial Nitric Oxide Synthase

Endothelial nitric oxide plays a large role in our bodies, primarily being a mediator of angiogenesis, the formation of new blood vessels. Nitric oxide is an endothelial "survival factor," and as such, it inhibits apoptosis and enhances endothelial cell proliferation/migration. As a vasodilator, it increases blood flow and leads to inflammation within our bodies (Cook and Losordo, 2002). Nitric Oxide is synthesized by the Nitric Oxide Synthanse (NOS). It is a complex enzyme that incorporates oxygen, arginine, and NADPH (nicotinamide adenine dinucleotide phosphate) to yield nitric oxide, citrulline and NADP+. This reaction is catalyzed by five separate cofactors: FMN, FAD, Heme, calmodulin, and tetrahydrobiopterin (Figure 3). This process shows NOS's involvement with the body's natural metabolism, particularly the Citric Acid Cycle as it lies at the end of several system processes such as the Renin-Angiotensin System and the Kallikrein-Kinin System.

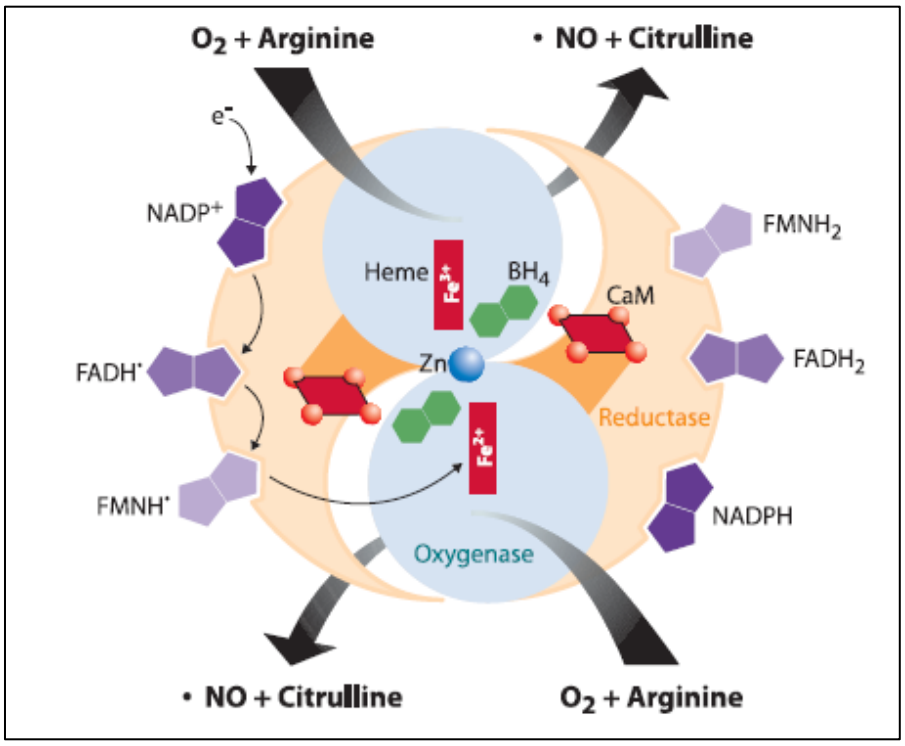


Figure 3: Nitric Oxide Synthesis (Cayman Chemicals)

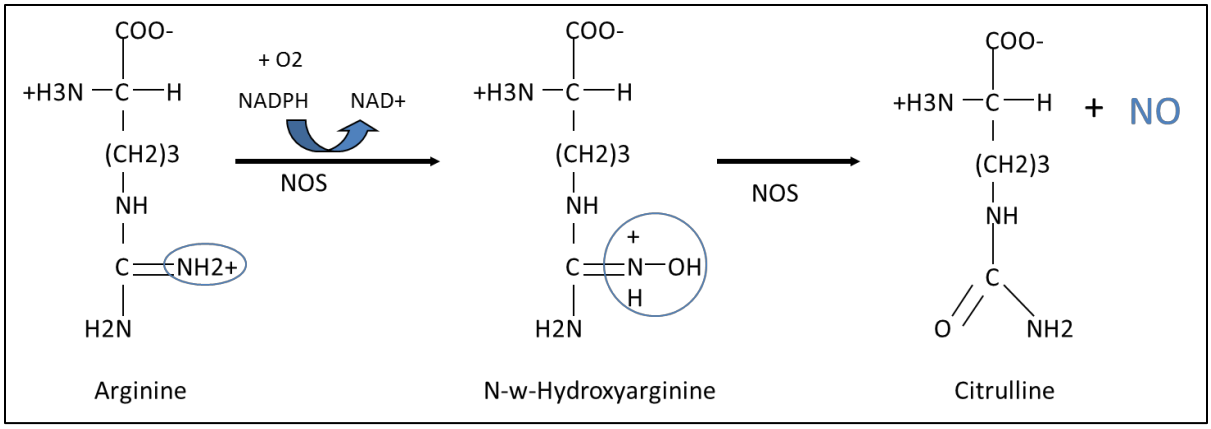


Figure 3.1: Nitric Oxide Synthesis

F) Plasma Kallikrein Kinin System

To understand more about nitric oxide, it is beneficial to understand the relationship between the vascular Kallikrein-Kinin System and blood pressure regulation. Plasma kallikrein is a serine protease that liberates kinin from kininogens, plasma proteins which are converted into active peptides. The best known kinin, bradykinin, is our main focus when it comes to blood pressure regulation (Hillmeister and Persson, 2012). Bradykinin acts via two different receptors, bradykinin receptor one and two (B1R & B2R). Comprised by kallikrein, kininogen, kinins, and receptors, the Kallikrein-Kinin System (KKS) is a complex, interweaving system. Besides the discovery of its role in renal pathophysiology, the Kallikrein-Kinin System is important to understanding blood pressure homeostasis and the pathogenesis of hypertension, and other cardiovascular diseases (Hillmeister and Persson, 2012).

The plasma kinin-forming system, also known as the the Kallikrein-Kinin System (KKS), is comprised of three proteins: coagulation Factor XII, prekallikrien (PK), and high molecular weight kininogen (HK). Before the Kallikrein-Kinin System can activate, high molecular weight kininogen (HK) must bind to the endothelial cell surface. HK is a multifunctional, multidomain β-globulin protein that has a molecular weight of 120 kD, which is important when it is digested. In order for

HK to bind to the endothelial cell surface that, a binding site is needed, and when it comes to KKS and associated pathways, three binding sites have been found to be specific to endothelial cells (Kaplan and Ghebrehiwet, 2010). First is qC1qR, the receptor for the globular heads of C1q, the first component of the complement classical pathway. Besides gC1qR, there is also cytokeratin 1 and the urokinase plasminogen activator receptor (u-PAR). These receptors exist mostly as bimolecular complexes, but gC1qR can function in an uncomplexed form. Before the KKS can assemble on the endothelial cell surface, these surface structures must be highly, negatively charged, and the HK must have changed to the proper conformation via zinc (Zn^{2+}) activation (Kaplan and Ghebrehiwet, 2010).

It should be pointed out that the KKS is not the only system that benefits from these binding complexes; the coagulation pathway and complement pathway are intertwined with the KKS. The KKS begins as HK binds to the gC1qR-cytokeratin 1 bimolecular complex. Meanwhile, Factor XII binds, temporarily, to the cytokeratin 1-u-PAR bimolecular complex long enough to go through autocatalytic activation. This activation converts Factor XII into Factor XIIa, activating a positive amplification mechanism to yield more Factor XIIa from Factor XII, but as a side effect, plasma prekallikrein (PK) is converted into plasma kallikrein (Ghebrehiwet and Peerschke, 2006). The newly convered kallikrein goes on to digest the HK in order to produce bradykinin. After moving the KKS pathway forward, Factor XIIa continues down its own coagulation pathway to its next substrate, coagulation Factor XI (Figure 4). These proteins interact through contact, activating multiple processes in a cascade and creating bradykinin as a cleavage product (Ghebrehiwet and Peerschke, 2006).

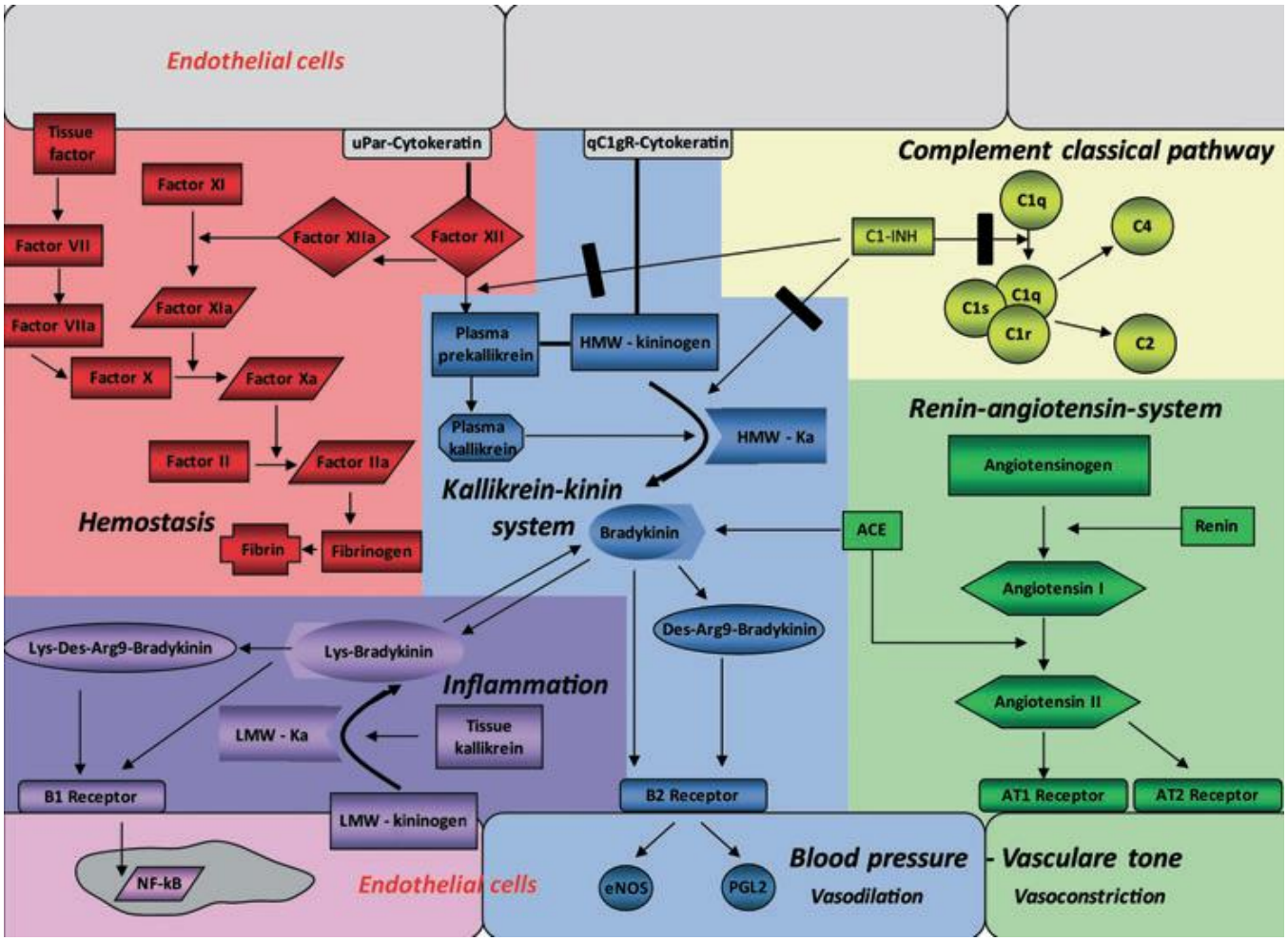


Figure 4: This diagram shows the Kallikrein-Kinin System's, along with the Coagulation Cascade, Renin-Angiotensin System, and Complement Classical Pathways', roles in blood pressure regulation (Hillmeister and Persson, 2012).

G) Bradykinin and its Receptors

As a significant part of the inflammatory process, bradykinin (BK) has been observed to have vasodilatatory effects, hinting that it plays a protective role in the cardiovascular system. Specifically, BK induces vasodilation through the release of mediators from the endothelial cell surface, nitric oxide (NO) and prostacyclin (PGI_2) (Donnini et al., 2010). Bradykinin has two receptors, both of which are mediated differently. The B_2 kinin-receptor (B2R) is a G-protein-coupled receptor that is constantly expressed in a great quantity of human organs and tissues besides the endothelium. Through the vasodilative products of B2R, blood pressure can be regulated. On the other hand, the B_1 kinin-receptor (B1R) is seem to be inactive under standard conditions, only to be expressed in the presence of bacterial endotoxins and/or cytokines, frequently hinting towards the existence of an infection (Dendorfer et al., 1999). BK is an invaluable tool when it comes to blood pressure homeostasis.

H) Prolylcarboxypeptidase

One of the more poorly-understood aspects of blood vessel homeostasis is the protein prolylcarboxypeptidase (PRCP). This serine protease, much like Factor XII, produces plasma kallikrein from plasma prekallikrein. PRCP was originally characterized as a peptidase that cleaves small, carboxy terminus peptides at neutral pH, but many more functions were discovered later. In addition, PRCP also metabolizes the interaction between BK and angiotensin II (Hagedorn, 2011). Only a few experiments have uncovered vital information about PRCP. In a study on the hypothalamus, it was observed that the melanocortin system is affected by PRCP. Multiple studies have shown that mice with a PRCP enzyme deficiency have elevated melanocortin (α-MSH), leading to decreased food intake and weight loss. Normally, insulin (from the pancreas), and/or leptin (from

adipose tissue) act on anorexigenic neurosecretry cells in order to release α-MSH. This causes neuronal signals to tell the body to “eat less and metabolize more fuel (Nelson, 2008). However, in the presence of PRCP, α-MSH is inhibited, suggesting that PRCP can be stimulate appetit. Normally, the anorexigenic α-MSH signal, stimulated from leptin and insulin, would signal the body to stop eating and store energy. This works so to balance out food intake and energy expenditure in the presence of the orexigenic “NPY” signal, which tells the body to eat more (Figure 5).

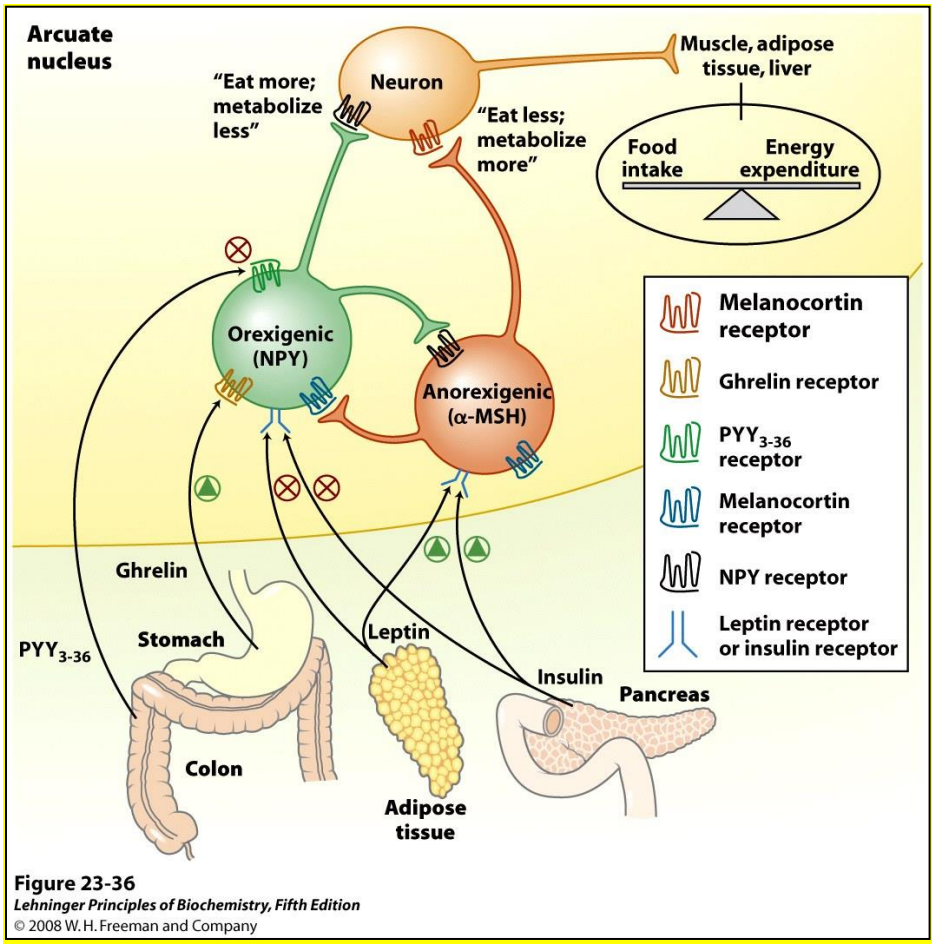


Figure 5: This diagram shows the arcuate nucleus (of the hypothalamus) and the process with which out food intake is balanced with our energy expenditure (Nelson, 2008).

In addition to the suggestion that PRCP functions as an appetite stimulant, it has been proven to have a large role in blood pressure regulation. Initially, PRCP was found to be a catalyst in the Renin-Angiotensin System (RAS), which also overlaps with the KKS. In renal tissues, PRCP controlled the amount of angiotensin II (Ang II) (Shariat-Madar et al., 2010). PRCP actively metabolized Ang II to angiotensin 1-7 (Ang 1-7), and the subsequent activation of the Ang 1-7 receptor (a G-protein-coupled receptor similar to bradykinin's receptors) would yield nitric oxide and prostaglandins. Meanwhile, the inactivation of α-MSH by PRCP also increases the production of proinflammatory molecules and promotes orexigenic action (Figure 6). Due to the negative feedback of Ang 1-7 countering the function of Ang II, it is believed that PRCP can minimize the negative, vasoconstritive effects of Ang II, diminishing the occurrence of cardiovascular diseases (Shariat-Madar et al., 2010).

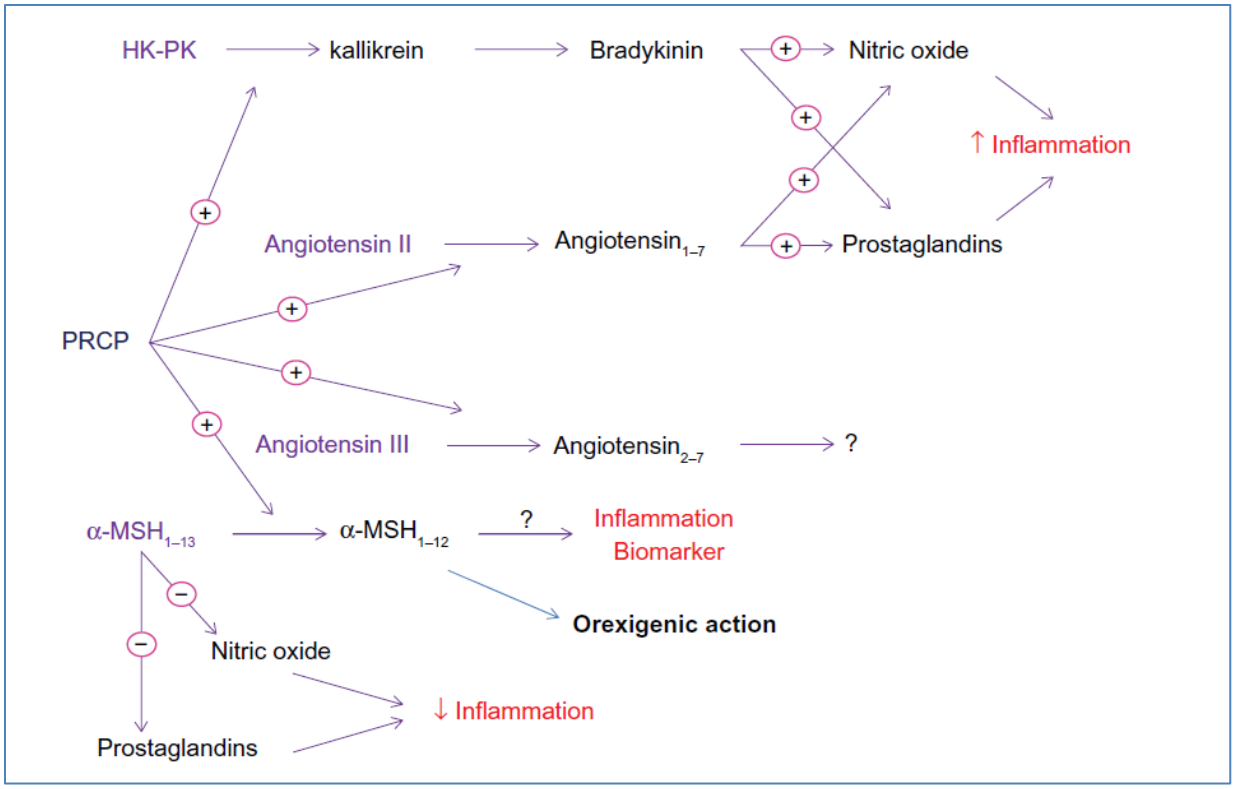


Figure 6: This diagram shows the pathophysiological actions of the serine protease, PRCP

I) B-galactosidase

Senescent cells in animals expressed increased levels of all proinflammatory molecules. This is interesting because it insinuates that cellular senescence contributes to the pathogenesis of atherosclerosis (Hayshi, et al., 2006). In order to better tie all of this together, it is important to look at the activity of β-galactosidase, a widely-accepted biomarker for cellular senescence. This active enzyme is detected using X-gal, which stains senescent cells blue in the presence of β-galactosidase. Acidic B-galactosidase is a eukaryotic hydrolase that is localized within the lysosome of cells. Because of this, its optimal processing conditions are around a pH of 4.5, although it can function between a pHs of 4 and 6. (Kurz et al., 2000).

J) Fibroblasts and Growth Factor #2

When looking at endothelial cells, it is important to keep fibroblasts in mind. Fibroblasts synthesize stroma, which acts as framework, for mammalian endothelium. It does so by manufacturing collagen, and the extracellular matrix (Ornitz and Itoh, 2001). Fibroblast Growth Factor-2 (FGF-2), the alkaline fibroblast growth factor, promotes wound healing and resides primarily in the subendothlial layer of blood vessels, making it a perfect target for observation in the HPAEC cell line. As FGF-2 has been seen playing a central role in mediating angiogenesis, it has also been observed having a rescuing effect on impaired endothelium. Much like eNOS and hTERT, the up-regulation of FGF-2 has been seen to favor cellular survival and delay endothelial cell senescence (Donnini et al., 2010).

K) Experimental Motivation and Reasoning

Many factors play into endothelial growth and eventual senescence. The up-regulation of various pathways and their downstream products prolong the life of the average cell. Telomerase decreases far before senescence sets in. PRCP, FGF-2, and eNOS all reach a peak of activity where

their angiogenic effects combat cellular aging and death. The exact etiology of endothelial senescence is still generally unclear; however, studies on many genetic components, and their regulation, have elucidated the process of degradation.

II) Materials & Methods

Endothelial Cell Culture – Human pulmonary artery endothelial cells (HPAEC) were grown and subcultured over time, from Passage 7 to Passage 40. HPAEC were obtained and cultured in endothelial growth medium (EGM) from Passage 7 to Passage 40. Cells were allowed three days to proliferate before each subculture procedure using Trypsin, DPSB, and the trypsin neutralizing solution (TNS). Right before separating the cells from their flask to be subcultured, the growth medium was aliquoted and saved. The cells were grown in fetal bovine serum (FBS) supplemented EGM at a constant temperature of 36.7°C, in an environment with a constant carbon dioxide level of 5%. The HPAEC, EGM, FBS growth supplement, HEPES, trypsin-EDTA, and trypsin neutralizing buffer were purchased from Clonetics (San Diego, CA).

HK/PK Activity – After obtaining a cell pellet from the trypsinized flask, the HPAEC are counted and calculated to ensure 30,000 cells per 100 µL well. After incubating the new cells in the 96-well plate, overnight, they were washed with HEPES and incubated for one hour with a 1% HEPES-gelatin mixture to block the plate. The plate was washed twice with HEPES again before adding, and incubating with, 20 nM HK for one hour. The wells were washed and treated with 20 nM PK before being incubated for another hour. Finally, the cells were treated with 0.5 mM a HD-Pro-Phe-Arg-paranitroanilide substrate (S2302) and incubated for an hour. All incubations took place at a constant temperature of 36.7°C, in an environment with a constant carbon dioxide level of 5%. The HK and PK were purchased from Enzyme Research Laboratory (South Bend, IN). S2302 was purchased from DiaPharma (Franklin, OH). After constructing the HK-PK Complex on recently subcultured cells in a 96-well plate, the activity of plasma kallikrein was quantified by detecting the amount of free paranitroanilide in vitro, at 405 nm using the ELx800 Plate Reader (Bio-Tek).

Western Blot – Adherent cell monolayer were washed with DPSB. The protein cell lysates were extracted using the RiPA Buffer (Thermo Scientific) and separated by electrophoresis in a 10% acrylamide gel; the standard used was the "Precision Plus Protein" standard, Dual Color from Bio-Rad (Cat. 161-0374). After electrophoresis, the gel was transferred to a nitrocellulose membrane in a buffer consisting of methanol, diH2O, and 10x Tris/glycine buffer at 4 °C for one hour. Once transferred, the membrane was blocked for one hour with 5% non-fat, dry milk in PBS with 0.1% Tween-20 (PBST). Then, the membrane is treated overnight at 4 °C with the primary antibody, Goat

Anti-Human PRCP (Bioscience), made in a 1:20 dilution. The next day, the membrane was washed thrice with PBST and then treated with a secondary antibody, Anti-Goat IgG: Whole Molecule, Peroxidase Conjugate (Bioscience), made in a 1:1000 dilution. The membrane was washed thrice again with PBST before being treated with the "Super Signal West Femto Maximum Sensitivity Substrate," courtesy of Thermo Scientific (Rockford, IL), a chemiluminescent substrate to be used for imaging with the ChemiDoc Imager (Bio-Rad).

Following the first set of imaging, the membrane was washed with 1x PBS and stripped with Thermo Scientific's "Restore Western Blot Stripping Buffer" at room temperature for 15 minutes before being blocked again and treated with the primary antibody, Mouse Anti-Human B-Actin (Santa Cruz, Cat. sc-49272) made in a 1:100 dilution, and the secondary antibody, was Anti-Goat IgG: Whole Molecule, Peroxidase Conjugate (Bioscience) made in a 1:1000 dilution. After blocking, treating, and washing, the membrane was imaged again and a ratio between the two densities was calculated.

Nitrite/Nitrate Assay – Growth medium was collected and stored over the course of growing HPAEC passages. The "Nitrate/Nitrite Fluorometric Assay Kit" (Cayman Chemicals) was used to quantify the metabolites of endothelial nitric oxide. Samples of used growth medium were adjusted to 80 µL with a 50/50 mix of fresh growth medium and Assay Buffer. Enzyme Cofactors and Nitrate Reductase was added to each well and the plate was incubated for an hour at room temperature. Afterwards, DAN (2,3-diaminoaphthalene) was added, followed by sodium hydroxide. The plate was read, and using the standard curve, the concentration of Nitrate + Nitrite was calculated.

Cellular Senescence Assay Kit – The "Chemicon International" Cellular Senescence Assay Kit was used on various passages of growing HPAEC cells to qualify their percentage of senescent cells. Senescent-associated β-galactosidase (SA-β-gal) is only present in senescent cells, and Chemicon's kit provides all reagents needed to detect SA-β-gal activity at pH 6.0 in cell cultures. SA-β-gal catalyzes the hydrolysis of X-gal, causing the accumulation of the blue dye in senescent cells.

Reverse-Transcriptase Polymerase Chain Reaction and Agarose Gel – Cell pellets were resuspended using TRIzol (Life Technologies). The mRNA was extracted from subcultured HPAEC using QIAGEN's "RNase-Free DNase Set." RNA was put through reverse-transcription via Invitrogen's "SuperScript III One-Step RT-PCR (with Platinum Taq) Kit." We bought multiple human DNA primers

from Invitrogen, including hTERT (SENSE – 5’ ATG GGG ACA TGG AGA ACA AG 3’ and ANTISENSE – 5’ GTG AAC CTG CGG AAG ACG GT 3’), B-Actin (SENSE – 5’ TGA ATG GAC AGC CAT CAT GGA C 3’ and ANTISENSE – 5’ TCT CAA GTC AGT GTA CAG GAA AGC 3’), FGF-2 (SENSE – 5’ TCA GCT CTT AGC AGA CAT TGG AAG AAA AAG 3’ and ANTISENSE – 5’ GGA GTG TGT GCT AAC CGT TAC CTG GCT ATG 3’), PRCP (SENSE – 5’ GTG GCT GAG GAA CTG AAA GC 3’ and ANTISENSE – 5’ TGT CAC CAA AGG GGA GAG AC 3’) , and eNOS (SENSE – 5’ ATG TTT GTC TGC GGC GAT GTT AC 3’ and ANTISENSE – 5’ ATG CGG CTT GTC ACT TCC TG 3’). The agarose gel varied in concentration from 1% to 2% depending on the primer under observation. We purchased Agarose powder from Invitrogen and ethidium bromide from Sigma.

III) EXPERIMENTATIONS

A) Plasma Kallikrein Activity

As Human Pulmonary Artery Endothelial Cells (HPAEC) Age, Plasma Kallikrein (PK) Activity Increases, Until Reaching a Peak before Tapering Off – The binding of high molecular weight kininogen (HK) has been described previously. Because the Kallikrein-Kinin System (KKS) has been discovered to be linked to regulation of blood pressure, among many other protective effects, we decided to measure the activation of PK from the complex of HK and plasma prekallikrein by detecting the absorbance of freed PNA from S2302 (Dendorfer, Wolfrun, & and Dominiak, 1999). The HPAEC were subcultured repeatedly, from Passage 7 to Passage 40 in order to show a gradual decrease into cellular senescence. Our results yielded that PK Activity does increase over time, peaking at Passage #18. After Passage 18, the PK activity decreases, gradually.

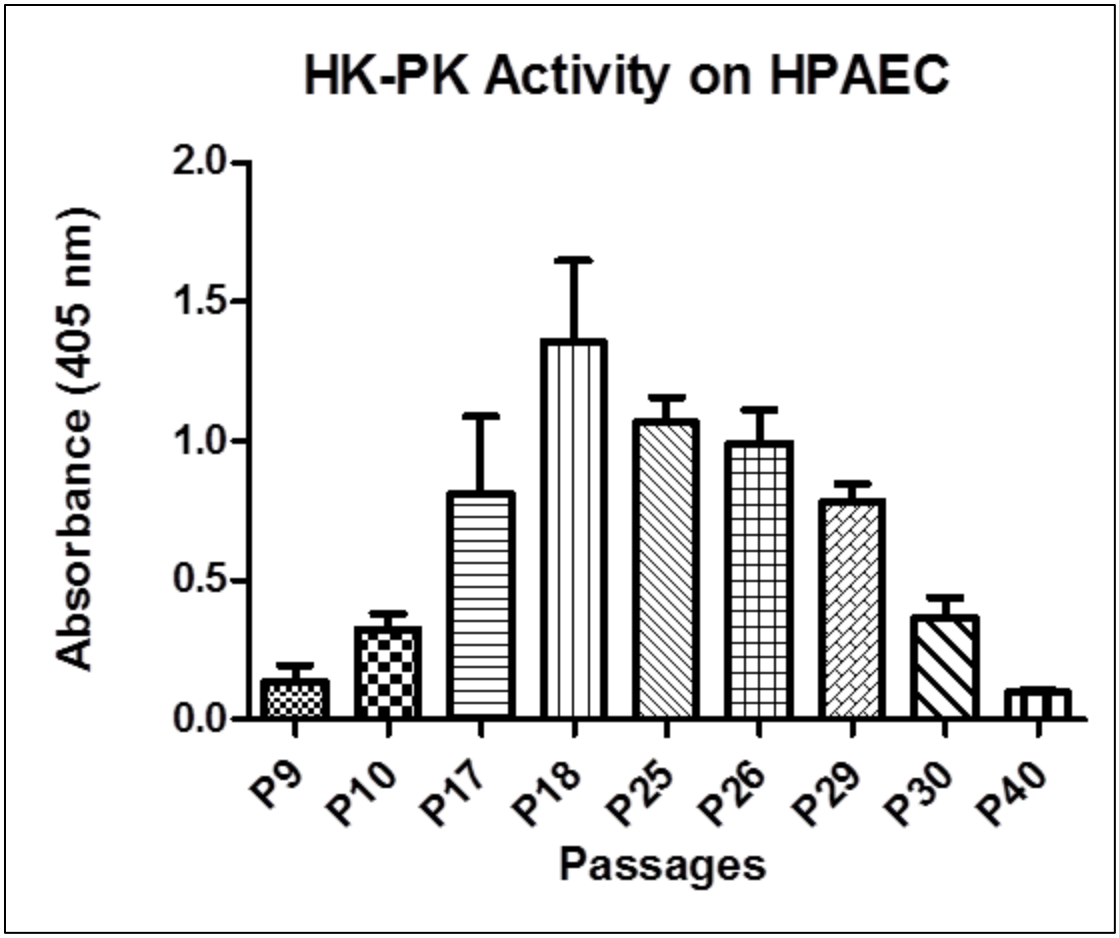


Figure 7: The formation of Plasma Kallikrein is measured from the High Molecular Weight Kininogen-Plasma Prekallikrein Complex. The absorbance of paranitroanilide, cleaved by activated kallikrein, was measured at 405 nanometers.

We know that Factor XII catalyzes the release of PK from the HK-PK Complex, but so does prolylcarboxypeptidase (PRCP). We can infer that the trend exhibited by the HK-PK activity should match the trend of PRCP activity.

B) PRCP Protein Expression

PRCP Expression Coincides with HK-PK Activation – After performing multiple Western Blots to measure protein expression of PRCP, it was found that a trend similar to HK-PK Activity can be found. PRCP expression increases from Passages 11 and 17 to peak at Passages 18 and 19, followed by a significant decrease in Passage 25 (Figure 8).

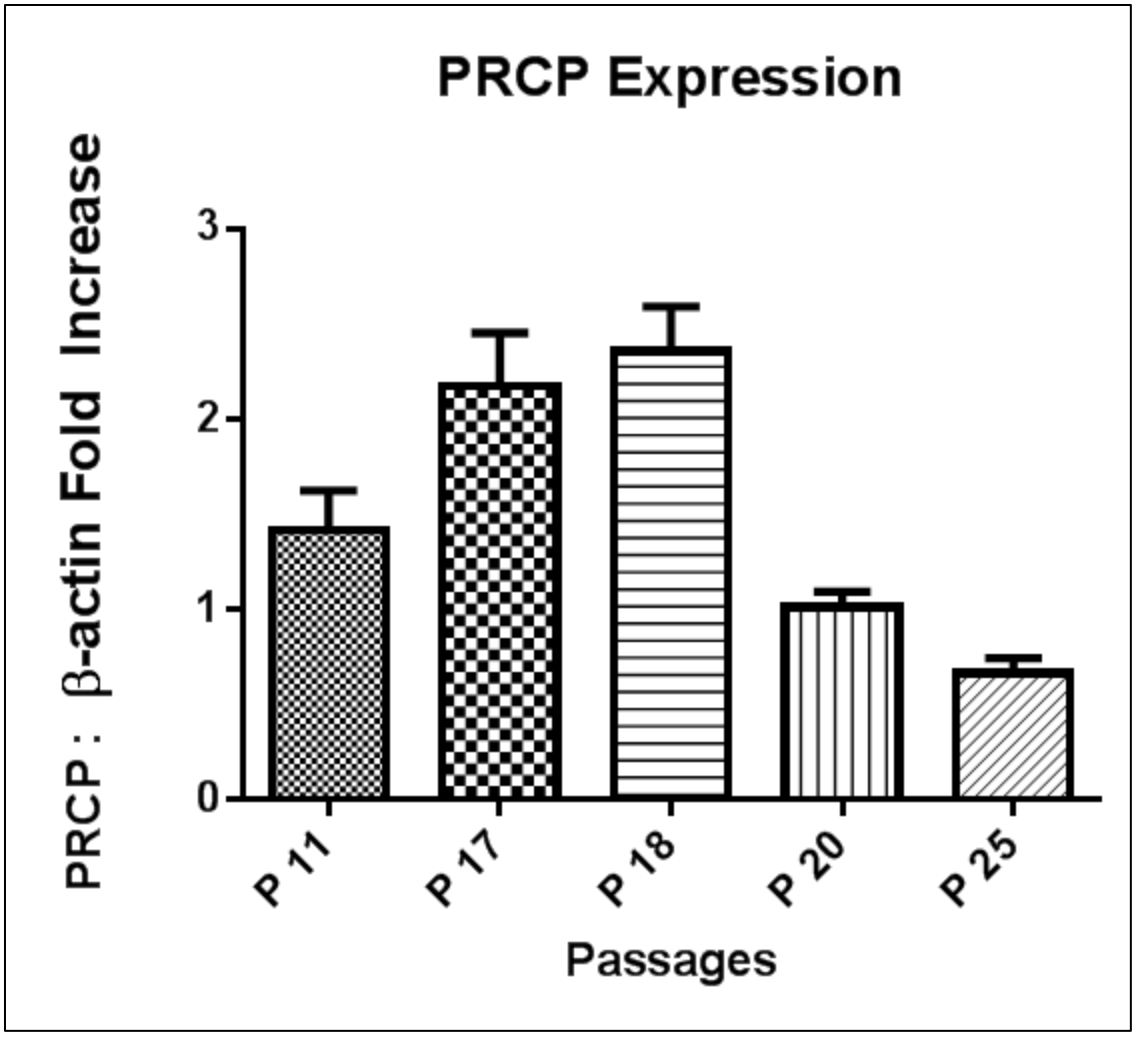
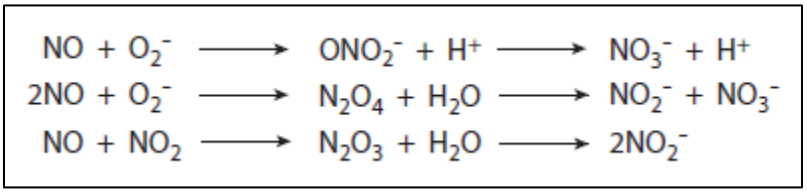


Figure 8: First, PRCP Expression was measured in various HPAEC. The expression of the target protein was quantified and then a ratio was normalized between PRCP and a cellular structure, control protein β-actin.

This trend of PRCP expression is logical due to the fact that one of PRCP's many roles is to produce PK from plasma prekallikrein (Peerschke, 2006). After treating and imaging the membrane with anti-PRCP, the membrane was stripped and treated with anti-B-Actin, in order to provide a normalized and accurate set of results.

C) Nitric Oxide Product Fluorometry

Nitric Oxide Detection via Fluorometric Assay Agrees with PRCP Expression – The final products of NO, in vivo, are nitrite (NO_2^-) and nitrate (NO_3^-) (Figure 9). Due to the fact that they are produced in random proportions, the most accurate way to measure total NO production is to measure the sum of both nitrate and nitrite. The NO quantification can be completed in two steps. First, nitrate is converted to nitrite via nitrate reductase. Second, acidic DAN (2,3-diaminonaphthalene) is added. Sodium hydroxide, which enhances detection of 1(H)-naphthotriazole (the fluorescent product), is added last (Figure 10).



IN VIVO:

1) Equal parts nitric oxide reacts and superoxide ion to yeild peroxynitrite, an unstable structural isomer of nitrate.
2) Two parts nitric oxide react with one part superoxide ion to yield dinitrogen tetroxide, which reacts with water to yield both nitrous acid and nitric acid. These acids are then reduced to nitrate and nitrite.
3) Equal parts NO and nitrogen dioxide yields dinitrogen trioxide which reacts with water to yield two nitrite salts. These salts decompose, as a result of heat, into two parts nitrogen dioxide and one part sodium hydroxide

Figure 9: A series of simplified, unbalanced reactions involving NO and various molecules in vivo, and brief explanations of those reactions.

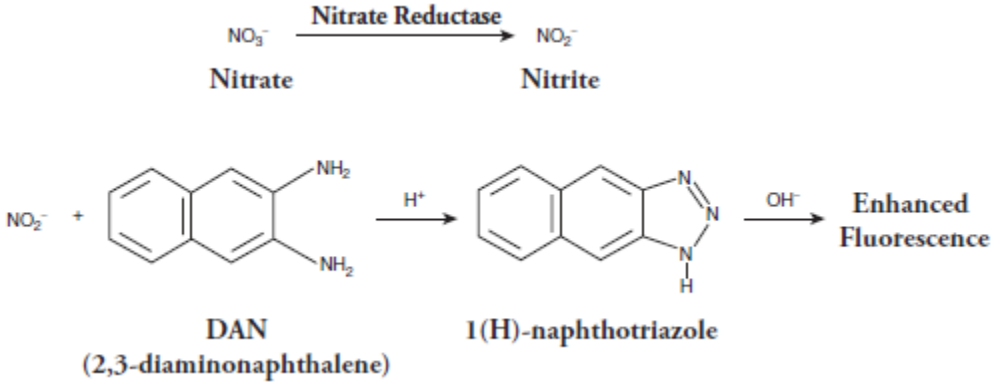


Figure 10: The chemistry behind the fluorescence of nitric oxide metabolites and their detection.

Nitric Oxide Production Mimics the Pattern of PRCP Activity –Understandably, as the cell passage increased, so did the amount of NO products. This supports the findings of the previous PRCP protein detection. As NO is a downstream product of PRCP, and a vasodilative, protective molecule, the peak would be expected around P17, 18, and 19, as is seen with PRCP (Figure 11).

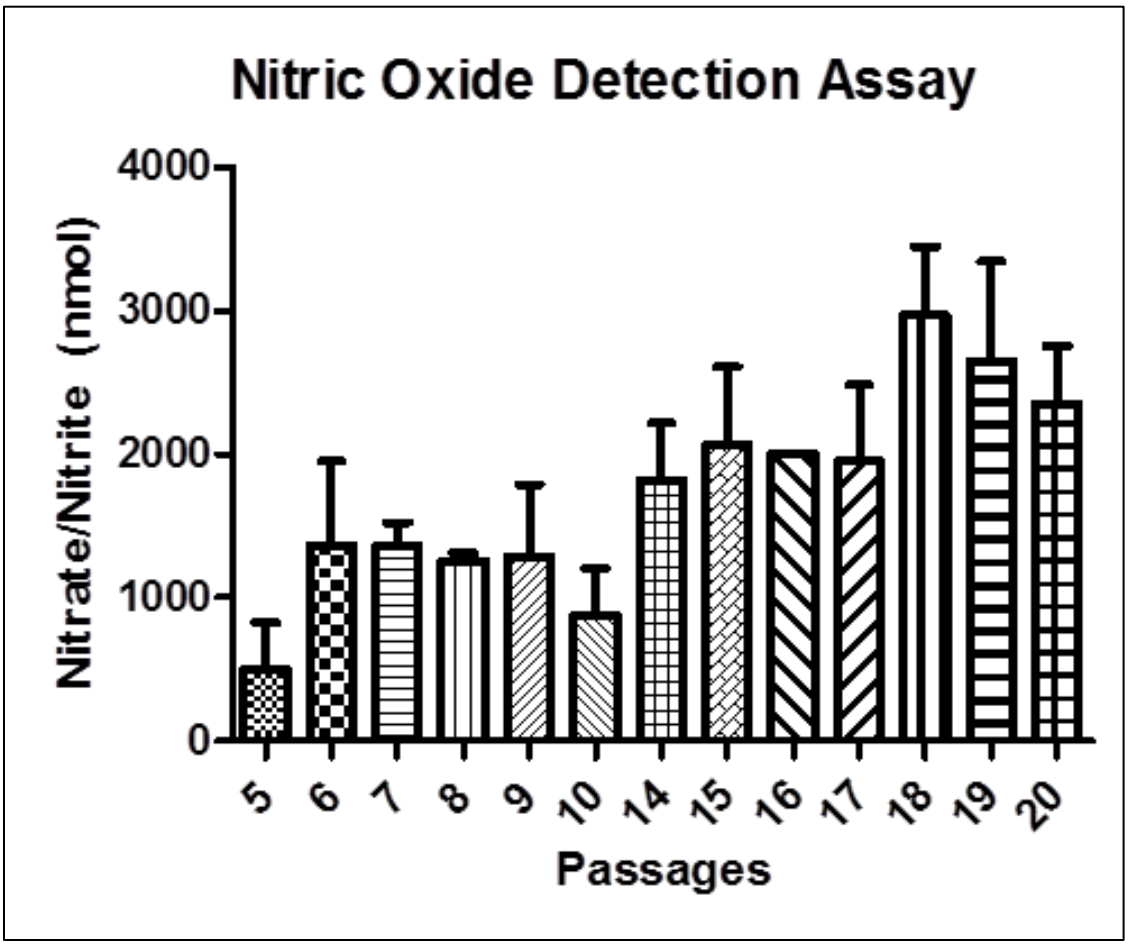


Figure 11: The products of nitric oxide, nitrate and nitrite, measured over time. Similarly to the quantification of PRCP, NO products also peak later before experiencing a drop in activity.

D) Endothelial Nitric Oxide Synthase DNA Expression

eNOS DNA Quantification Mimics NO Detection – After quantifying the amount of nitric oxide product, the next logical step would be to quanitify the amount of endothelial nitric oxide synthase (eNOS). After measuring the products of NO, mRNA from the same HPAEC cell lines was extracted and put through reverse transcriptase polymerase chain reaction (RT-PCR) with a primer for endothelial nitric oxide synthase. This would enable us to quantify the amount of eNOS present in cell passages over time. Our results supported previous findings with NO, showing that there is a rapid increase in eNOS over time, followed by a slow decrease (Figure 12). This slow decrease is due to the rising need for NO to oxidize the increased quantity of reactive oxygen species. As NO is rapidly used up, eNOS remains steady for several late, senescent passages

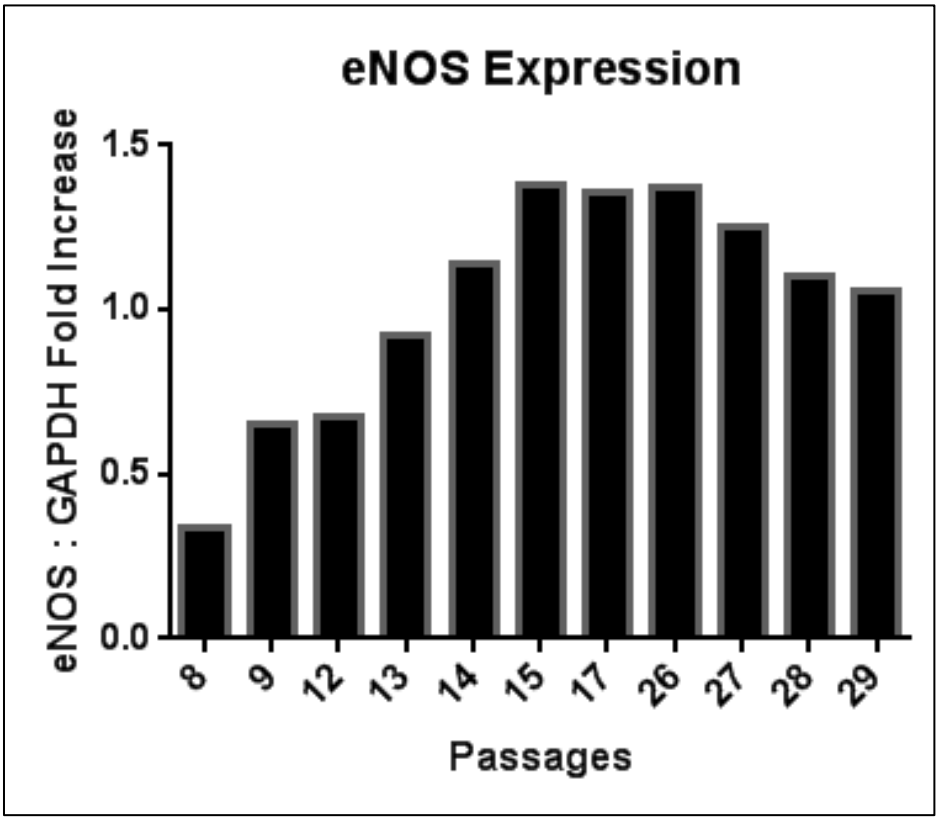
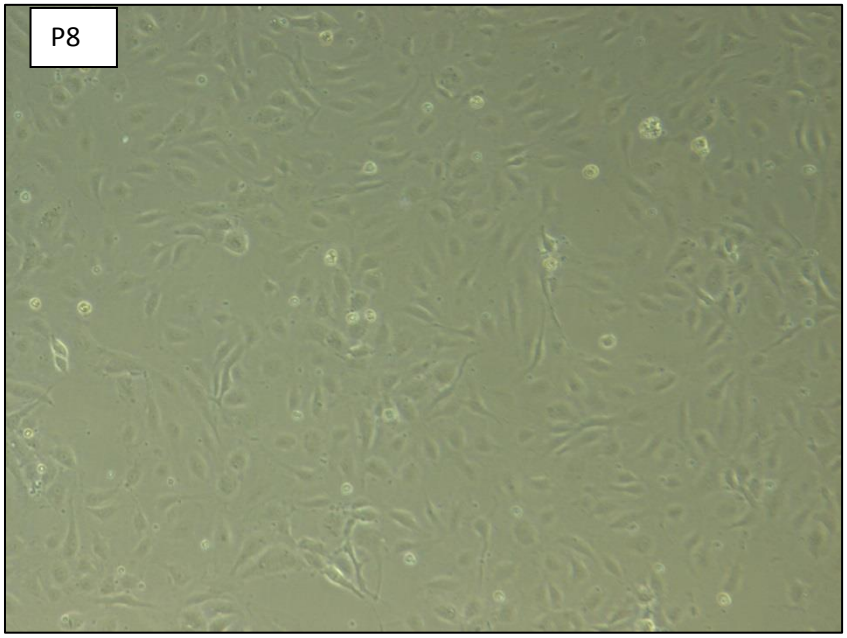
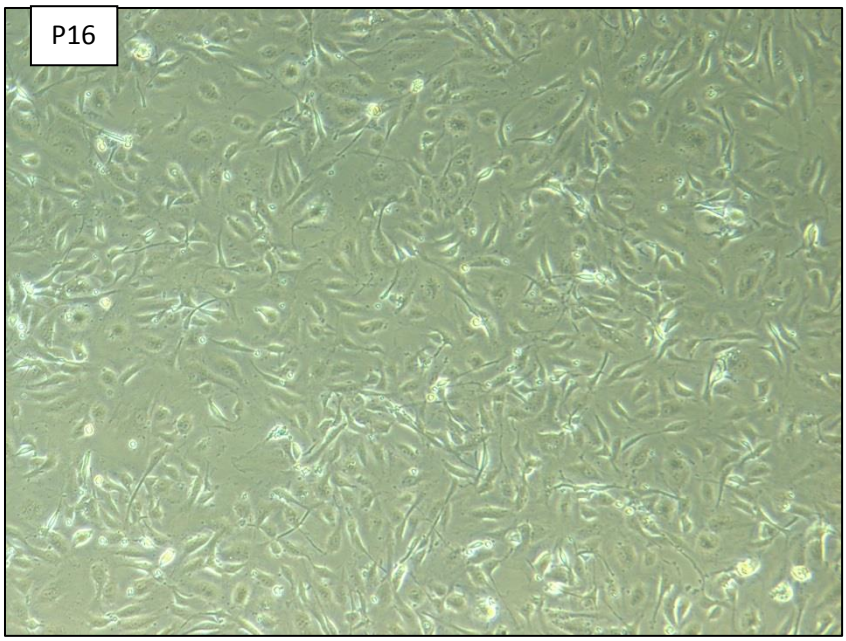


Figure 12: The sustained peak in eNOS supports the findings that nitric oxide delays endothelial cell senescence.

E) B-Galactosidase Staining and Relative Cell Count

B-Galactosidase Activity Increases With Age – In order to better visualize the effect of aging on endothelial cell senescence, β-Galactosidase staining was implemented on various HPAEC passages. As cells age, their concentration of B-galactosidase increases, as the staining reveals. Relative cell count was measured and graphed (Figure 13). As a universal marker for endothelial cell senescence, the increase in B-galactosidase is expected to coincide with a decrease in telomerase (hTERT).

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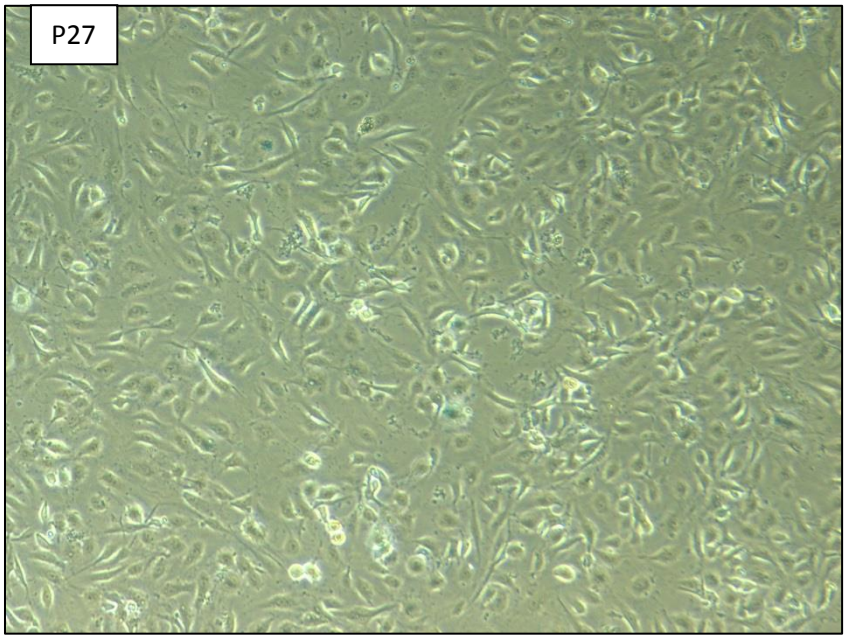
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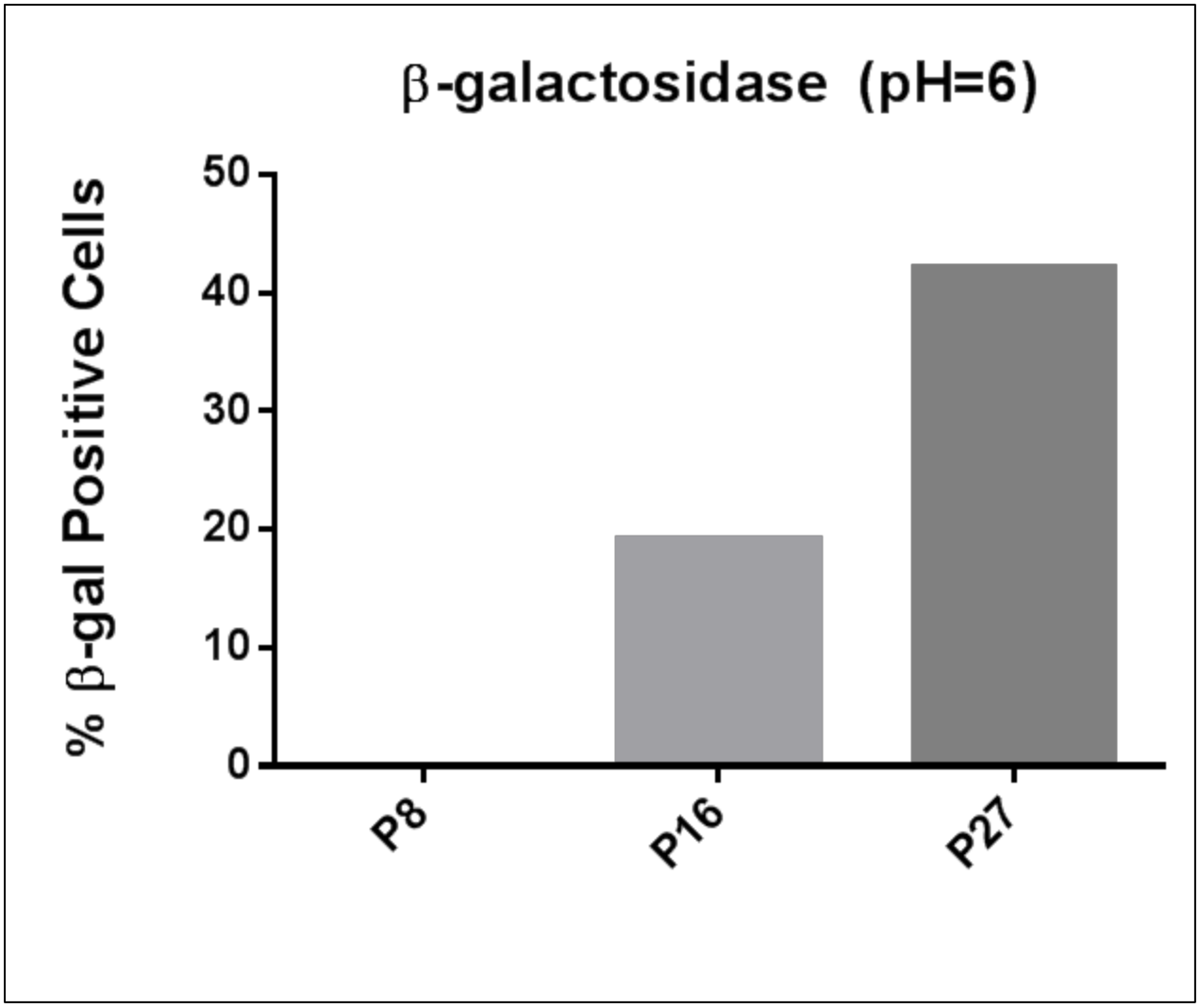

Figure 13: B-galactosidase staining was performed on various HPAEC cell passages. Using the microscope's camera, pictures were taken of each passage, and relative B-galactosidase-positive cell percentage was calculated.

F) Human Telomerase Reverse Transcriptase mRNA Expression

hTERT DNA Quantification Shows Steady Decrease Over Time – Senescence, detectable through B-galactosidase staining, is triggered by constant shortening of telomeres; therefore, it can be delayed by telomersase. After staining for B-galactosidase, mRNA from the same HPAEC cell lines was extracted and put through reverse transcriptase polymerase chain reaction (RT-PCR) with a primer for human telomerase reverse transcriptase. Our observations show that as hTERT decreases, B-galactosidase increases. In fact, no B-gal is detected until hTERT reaches minimal levels (Figure 14). It can be concluded that hTERT delays endothelial cell senescence via its function of elongation of telomeres.

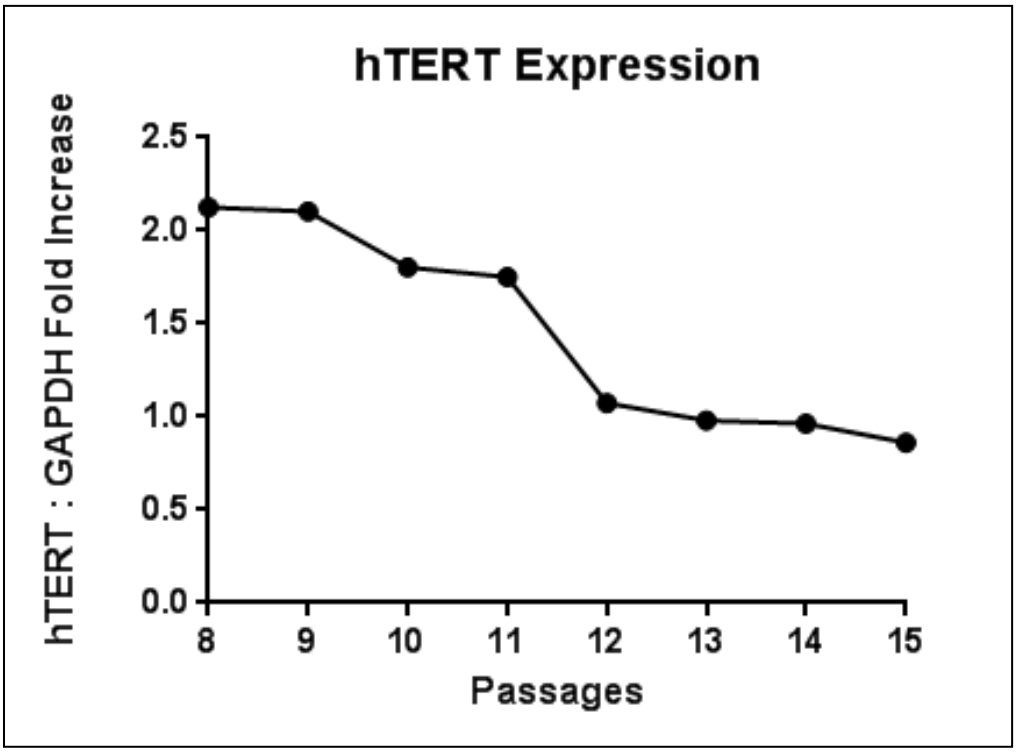

Figure 14: Telomerase decline precedes endothelial cell senescence.

G) Prolylcarboxpeptidas mRNA Expression

PRCP DNA Quantification Mimics PRCP Protein Quantification – In order to further look into PRCP, we extracted mRNA from the same cells that we extracted lysis to analyze the protein. We used RT-PCR and quantified the DNA. Our results show that PRCP follows the same trend as seen before, reaching maximum activity around Passages 17 and 18 (Figure 15).

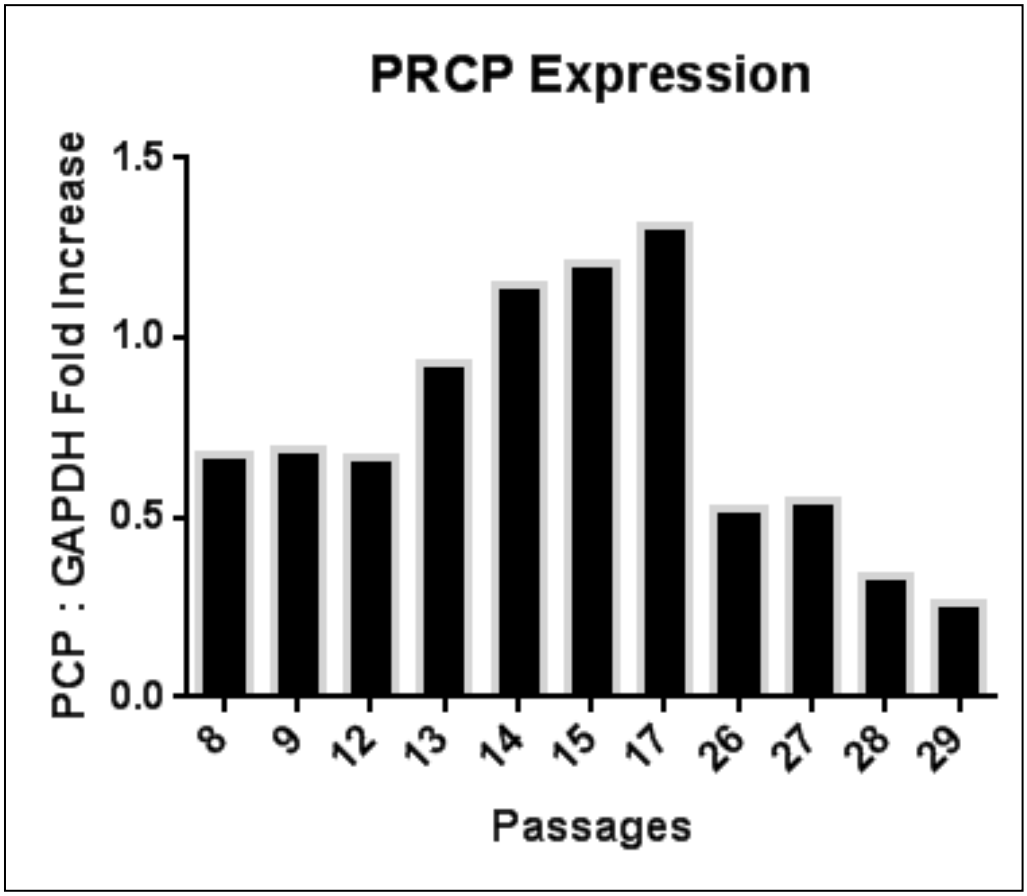

Figure 15: PRCP DNA follows the same trend as PRCP Protein, further supporting the evidence of PRCP playing a cardioprotective role via senescence delay.

H) Fibroblast Growth Factor-2 mRNA Expression

FGF-2 DNA Quantification Mimics PRCP – After looking into many facets of study pertaining to angiogenesis and cardiovascular health, it was a logical continuation to add a study of fibroblasts to the experiments. Fibroblast growth factor #2 is responsible for cell survival and the formation of new blood vessels. Keeping this in mind, we would expect it to flourish with the up-regulation of PRCP and other angiogenic molecules. FGF-2 mimicked PRCP, peaking slightly earlier, but followed the same trend as PRCP, reinforcing the belief that, it too, promotes cell survival and the delay of endothelial cell senescence (Figure 16.)

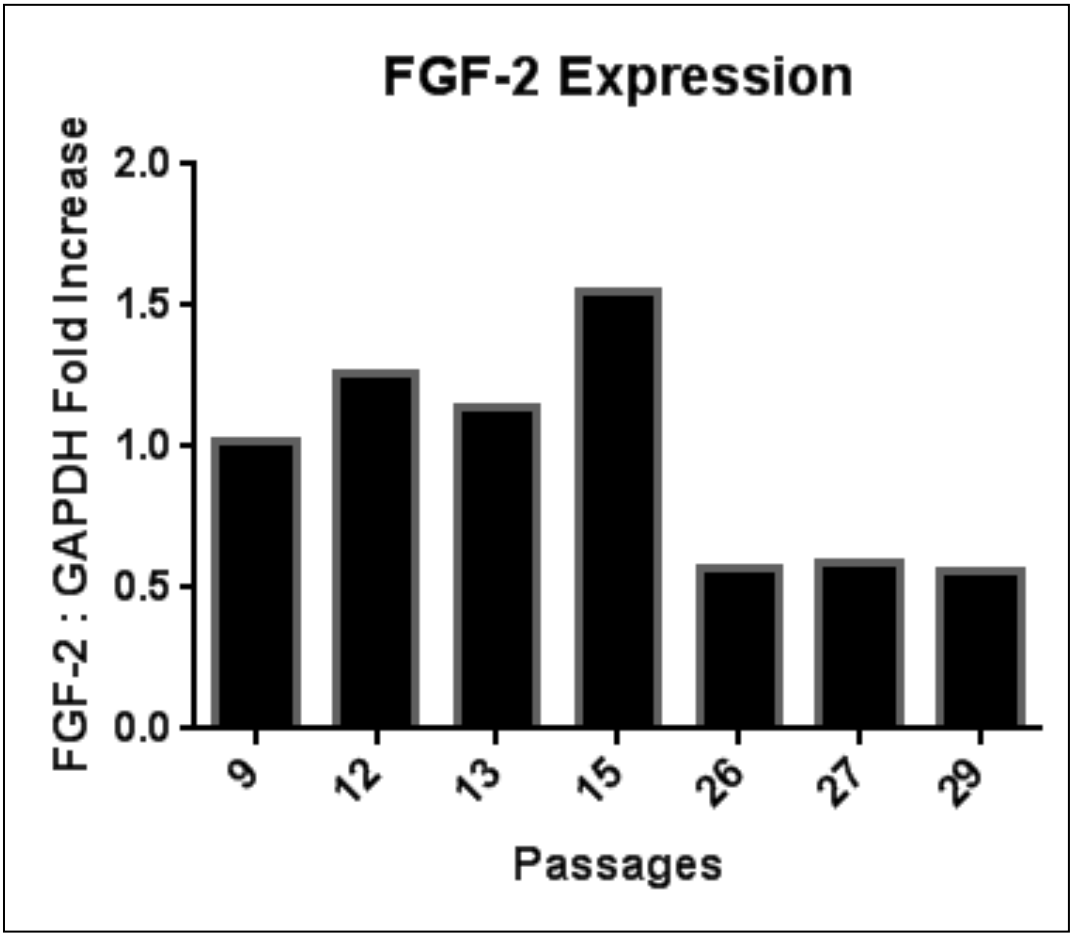

Figure 16: FGF-2 promotes cellular survival, proliferation, and angiogenesis

IV) DISCUSSION

The plasma kallikrein kinin system plays a major role in inflammation and blood pressure regulation via its unique blood proteins. By investigating the origin complex of this system, comprised of HK and PK, we found a great starting point to investigate cardioprotective effects on senescence. PRCP, found to promote cardiovascular protection through many facets of autophagy, was observed in multiple forms, and a causal relationship was found between kallikrein activity and PRCP. When measuring PRCP protein concentration, B-actin was used as a cellular control in order to provide normalization of our results. After investigating the origins of the KKS, we decided to follow the pathway to the end. Quantifying the NO product would be necessary. As a downstream product, the highly reactive NO was successfully quantified from old growth medium in the form of its metabolites. In order to verify the validity of our NO product, a second source of the molecule was observed, the B2R-mediated eNOS. Our findings show that as senescence onset occurs, the need for NO rises rapidly, and for a while, eNOS activity maintains its peak level of expression to meet that need; however, decreasing levels of arginine eventually render eNOS inactive.

As a universal marker for senescence, observation of β-galactosidase was crucial when observing the decrease in hTERT over time. We knew β -gal would increase over time, and previous articles supported the idea of hTERT degradation being the penultimate step to the onset of cellular senescence. Our findings support that hypothesis. In order to be sure, we used GAPDH, a cellular control, when measuring hTERT, to provide normalization of our results. The inclusion of FGF-2, due to being a major source of angiogenesis, incorporated variety and allowed us to observe other types of cardioprotective proteins. The results from the FGF-2 study supported our previous findings and showing a senescence inhibiting effect via the Passage 15 peak.

Our data shows that the PRCP-mediated kallikrein kinin pathway delays endothelial cell senescence by promoting cellular survival through generation of vasodilative molecules, like nitric

oxide. In addition, the increase in β-gal coincides with not only the progression of senescence, but also the peak, and subsequent decrease, in PRCP and FGF-2 expression patters. Also, our data supports the idea of the decrease in hTERT initiating a change in the PRCP-induced PK activation, leading to the onset of senescence. In conclusion, to the PRCP is believed to play a role in autophagy and blood protein activation in order to extend the life of endothelial cells. The interplaying relations ship between the kallikrein kinin system, renin angiotensin system, and other associated pathways is shown in Figure 17 (gC1qR = globular C1q receptor, CK1 = cytokeratin 1, uPAR = urokinase plasminogen activator receptor, PK = prekallikrein, HK = high molecular weight kininogen, KA = activated kallikrein, HKa = high molecular weight kiniogen a, PRCP = prolylcarboxypeptidase, BK = bradykinin, BK1-8 = des-Arg-bradykinin, KD = kallidin (lys-bradykinin), B2R = constitutive bradykinin receptor, eNOS = endothelial nitric oxide synthase, B1R = inducible bradykinin receptor, iNOS = inducible nitric oxide synthase, NO = nitric oxide, Arg = arginine, Cit = citrulline,, FGF-2 = fibroblast growth factor #2, hTERT = Human Telomerase Reverse Transcriptase (hTERT), α-MSH = alpha-melanocyte stimulating hormone, β-gal = beta-galactosidase, Ang = angiotensin, MR = mass receptors.)

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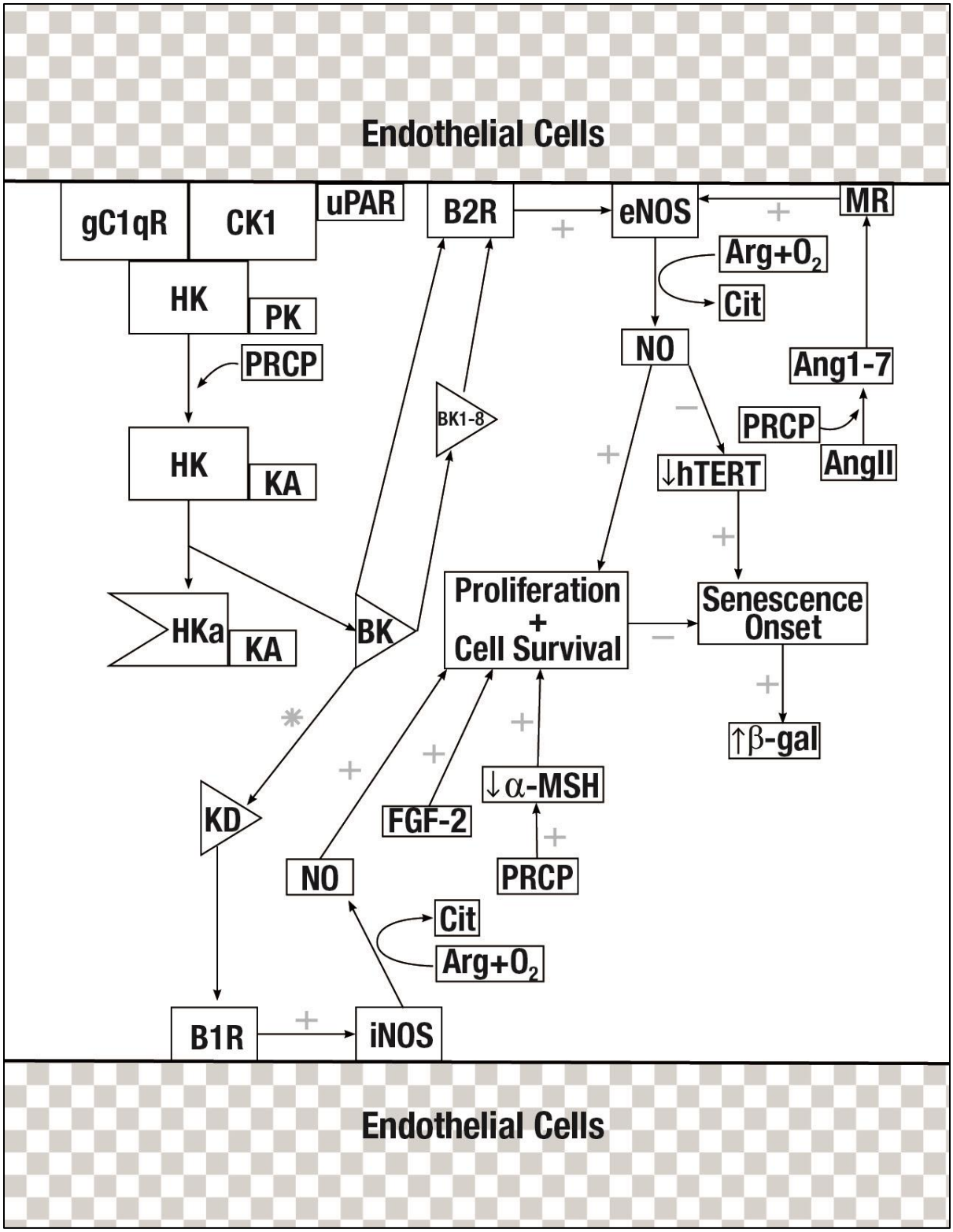

Figure 17: A chart summarizing the various causes of vasodilation, angiogenesis, and cell proliferation, and their involvement with the onset of endothelial cell senescence. (*→ induced by tissue damage/inflammation, + → activates, - → inhibits)

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V) WORKS CITED

McClintock, B. *Genetics* **1941,** *26,* 234-282.

Hayflick, L. *Experimental Cell Research* **1965,** *37,* 614-636.

Cooke, J. P.; Losordo, D. W. *Circulation* **2002,** *105,* 2133-2135.

Dendorfer, A.; Wolfrum, S.; Dominiak, P. *Japanese journal of pharmacology* **1999,** *79,* 403-426.

Fox, S. I. *Human Physiology, 6th ed.* MGraw-Hill: Boston, 1999.

Hagedorn, M. *Blood* **2011,** *117,* 3705-3706.

Hayashi, T.; Matsui-Hirai, H.; Miyazaki-Akita, A.; Fukatsu, A.; Funami, J.; Ding, Q.-F.; Kamalanathan, S.; Hattori, Y.; Ignarro, L. J.; Iguchi, A. *Proceedings of the National Academy of Sciences* **2006,***103,* 17018-17023.

Hillmeister, P.; Persson, P. B. *Acta Physiol* **2012,** *206,* 215-219.

Kaplan, A. P.; Ghebrehiwet, B. *Molecular immunology* **2010,** *47,* 2161-2169.

Aubert, G.; Lansdorp, P. M. *Physiological Reviews* **2008,** *88,* 557-579.

Nelson, D. L. *Lehninger Principles of Biochemistry, 5th ed.* W. H. Freeman and Company: New York, 2008.

Peerschke, E. I.; Ghebrehiwet, B. *Immunobiology* **2007,** *212,* 333-342.

Schmieder, R. E.; Hilgers, K. F.; Schlaich, M. P.; Schmidt, B. M. *Lancet* **2007,** *369,* 1208-1219.

Shariat-Madar, B.; Kolte, D.; Verlangieri, A.; Shariat-Madar, Z. *Diabetes, metabolic syndrome and obesity : targets and therapy* **2010,** *3,* 67-78.

Vasa, M.; Breitschopf, K.; Zeiher, A. M.; Dimmeler, S. *Circulation Research* **2000,** *87,* 540-542.

Donnini, S.; Terzuoli, E.; Ziche, M.; Morbidelli, L. *The Journal of pharmacology and experimental therapeutics* **2010,** *332,* 776-784.

Shen, X.-h.; Xu, S.-j.; Jin, C.-y.; Ding, F.; Zhou, Y.-c.; Fu, G.-s. *International Immunopharmacology* **2013,** *16,* 261-267.

Kurz, D. J.; Decary, S.; Hong, Y.; Erusalimsky, J. D. *Journal of cell science* **2000,** *113 (Pt 20),* 3613-3622.

Hayashi, T.; Kotani, H.; Yamaguchi, T.; Taguchi, K.; Iida, M.; Ina, K.; Maeda, M.; Kuzuya, M.; Hattori, Y.; Ignarro, L. J. *Proceedings of the National Academy of Sciences* **2014,** 2013-2153.

Ornitz, D. M.; Itoh, N. *Genome biology* **2001,** *2*.

Shampay, J.; Szostak, J. W.; Blackburn, E. H. *Nature* **1984,** *310,* 154-157.